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Influence of temperature and water activity on Ochratoxin A production by *Aspergillus* strain in coffee south of Minas Gerais/Brazil

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ABSTRACT

This study investigated the influence of temperature and water activity (a_w) on the growth and biosynthesis of Ochratoxin A (OTA) by *Aspergillus carbonarius* and *Aspergillus ochraceus*, and the influence of climate change on the production potential of this mycotoxin in coffee was predicted. Central Composite Rotatable Design with a_w ranging from 0.91 to 0.99 and temperatures from 17 to 38 °C was used. *A. carbonarius* CCDCA 10288 and 10293 showed optimal growth in a_w intervals from 0.935 to 0.965 and temperatures between 25 and 32 °C while *A. ochraceus* CCDCA 10211 and 10212 showed a_w intervals from 0.940 to 0.990 and temperatures between 21 and 30 °C. The conditions under which strains show the highest OTA risk index are as follows: for *A. carbonarius* CCDCA 10288 and 10293, a_w between 0.95 and 0.99 and temperatures between 22 and 32 °C, for *A. ochraceus* CCDCA 10211 a_w between 0.97 and 0.99 and temperatures between 25 and 30 °C. Using the models obtained, it was possible to verify that the conditions become more favorable for OTA production in the harvest months due to increased temperatures in the southern region of Minas Gerais. A safe product can be ensured by following good practices.

1. Introduction

Coffee beans may be contaminated by toxigenic fungi during processing, particularly during washing, fermentation, and drying (Silva, Batista, & Schwan, 2008; Taniwaki, Pitt, Teixeira, & Iamanaka, 2003). *Aspergillus carbonarius* and *A. ochraceus* are among the main molds that have been found in association with coffee, and they produce OTA (Batista, Chalfoun, Prado, Schwan, & Wheals, 2003; Taniwaki et al., 2003). Other species that produce OTA and that can be found in coffee include *A. steynii*, *A. westerdijkiae, A. niger*, and *A. sclerotiorum* (Gil-Serna et al., 2014; Joosten, Goetz, Pittet, Schellenberg, & Bucheli, 2001; Suárez-Quiroz et al., 2004; Urbano, Taniwaki, Leitão, & Vicentini, 2001).

Depending on the time and temperature used to roast coffee, OTA may not completely destroyed (Raters & Matissek, 2008) and may be masked by other compounds (Suárez-Quiroz et al., 2005). Another factor that may influence the concentration of OTA is the particle size of ground coffee (Oliveira et al., 2013). If the toxin is present in the green beans, it can remain in the roasted coffee at levels greater than those allowed by legislation.

The development and biosynthesis of OTA by toxigenic fungi depends on environmental factors. Temperature and water activity (a_w) play an important role in this biosynthesis (Kogkaki, Natskoulis, & Panagou, 2016). Any climatic change can affect food safety and mycotoxins are of foremost concern (Medina, Rodrıguez, & Magan, 2015; Miraglia et al., 2009; Paterson & Lima, 2010). *In vitro* data on fungal growth and mycotoxin production may not be directly related to the field or post-harvest situation but are useful for making basic assumptions (Paterson & Lima, 2011). The effect of these factors on mycotoxin production and predicting the influence of climate change on this production is more easily understood when models that simulate climatic scenarios are developed (Battilani, 2016; Fels-klerx et al., 2009).

Studies have been conducted to evaluate the influence of these factors on the growth and production of mycotoxins by filamentous fungi on different substrates, especially the interaction of these factors (Akbar & Magan, 2014; Casquete et al., 2018; Kogkaki et al., 2016; Passamani et al., 2014). These results are useful to assess the current potential risk or to make future predictions based on climate change

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(Akbar, Medina, & Magan, 2016).

Therefore, the present study was carried out to evaluate, *in vitro*, the influence of temperature and water activity on the growth and potential risk of OTA production by *A. carbonarius* and *A. ochraceus* and obtain a model to predict the production potential of OTA due to climate change.

2. Materials and methods

2.1. Fungal isolates

Four toxigenic isolates were used in this study (*A. carbonarius* CCDCA 10288 and 10293, and *A. ochraceus* CCDCA 10211 and 10212). These fungi have been isolated from coffee and were obtained from the Culture Collection of Microorganisms of the Department of Food Science (CCDCA) of the Federal University of Lavras, Brazil.

2.2. Central composite rotatable design (CCRD)

A central composite rotatable design (CCRD) was used for two independent variables in a 2^2 factorial design (four factorial trials, three central points, and four axial points) (Rodrigues & Iemma, 2009) and was used to evaluate the effect of abiotic factors on the OTA biosynthesis and growth of the *A. carbonarius* and *A. ochraceus* strains. The independent variables studied in the process were temperature (X1) and a_w (X2). The response was the fungal growth (mm) and OTA risk index (ORI). The coded variables were established with five levels (−1.41, −1, 0, 1, 1.41). The evaluated range of temperature was 17–38 °C and a_w 0.91 to 0.99, resulting in 11 assays. Table 1 shows the design matrix with coded and real values for each assay and results obtained. The evaluated range of temperature and a_w were defined based on previous studies (Chiotta, Sosa, Ponsone, & Chulze, 2015; Kapetanakou, Panagou, Gialitaki, Drosinos, & Skandamis, 2009; Pardo, Ramos, Sanchis, & Marín, 2005).

2.3. Medium preparation

A green coffee-based medium was employed in this study and was prepared following the methodology of Pardo et al. (2005) with modifications. In brief, 175 g of ground green coffee was placed in a clean bag-shaped cloth, dipped into 1 L of boiling distilled water and simmered for 60 min, always making up the volume of water when necessary. The liquid was filtered on hydrophilized gauze and 20 g of agar (Merck) was added.

The a_w of the medium was measured using an Aqua-lab CX-2 (Decagon Devices, Inc.) and the initial value was 0.99, modified with the addition of glycerol as described by Belli, Ramos, Coronas, Sanchis, and Marín (2005) at different levels (0.91–0.99) as provided by the CCRD.

2.4. Reactivation of isolates and inoculation of spores

Isolates were initially grown on Malt Extract Agar (MEA; malt extract, 20 g; peptone, 1 g; glucose, 20 g; bacteriological agar, 20 g; distilled water, 1000 ml) medium incubated for 7 days at 25 °C. Spore suspensions were prepared according to Passamani et al. (2014).

Petri dishes containing 20 ml of solidified medium (green coffee-based) at different a_w, were centrally inoculated with 100 µl of spore suspension and incubated at a range of temperature conditions between 17 and 33.4 °C as defined by the CCRD.

2.5. Fungal growth and OTA analysis

Petri dishes were examined during incubation and the colony diameter was measured in two at right angles with the aid of a digital pachometer. OTA was extracted and analyzed according to Passamani et al. (2014). The HPLC system was equipped with a fluorescence detector RF-10 AXL (λexc 330 nm; λem 460 nm) and an Agilent-Zorbax Eclipse XDB-C18 (4.6 × 250 mm, 5 µm) column was used.

2.6. OTA risk index calculation

The results of OTA production were evaluated in relation to the OTA risk index (ORI) proposed by Gil-Serna et al. (2014), which relates fungal growth and OTA production in agar plates. The ORI relates the fungal colony area at the end of the experiment (10 days of incubation) with OTA production ability in each condition. The equation for calculating ORI follows below.

OTA risk index (ORI) = log [OTA concentration (ng/mm^2) × Fungal colony surface (mm^2) +1].

2.7. Use of the predicted model to estimate ORI

The obtained model was used to estimate the ORI in the southern region of Minas Gerais, the largest coffee producing region responsible for 56% of the state production according to the harvest data from 2017 Companhia Nacional de Abastecimento (CONAB, 2017). The temperatures used in the models are the averages obtained each month of 2017 in the region, according to the data provided by the National Institute of Meteorology (INMET) (2017). Furthermore, optimistic and pessimistic projection temperatures were used, according to the sixth report of the Intergovernmental Panel on Climate Change (IPCC) on climate change

Table 1
Matrix design and results obtained from the growth and ORI assays for each isolate: *Aspergillus carbonarius* CCDCA 10288 and CCDCA 10293, and *Aspergillus ochraceus* CCDCA 10211 and CCDCA 10212.

Expt.	Temp. (°C)	a_w	growth (mm)				ORI			
			10288	10293	10211	10212	10288	10293	10211	10212
1	−1 (20)	−1 (0.92)	9.64	9.95	25.19	24.82	1.24	1.26	1.92	2.05
2	−1 (20)	1 (0.98)	31.78	32.60	75.08	72.77	3.05	3.95	3.16	4.34
3	1 (35)	−1 (0.92)	34.95	36.54	26.80	19.78	2.20	2.69	2.17	2.80
4	1 (35)	1 (0.98)	40.00	39.92	38.89	36.98	3.78	3.38	2.99	4.11
5	−1.41 (17)	0 (0.95)	25.00	26.11	32.15	36.68	3.07	2.90	0.00	0.00
6	1.41 (38)	0 (0.95)	53.70	50.08	0.00	0.00	2.64	2.99	0.00	0.00
7	0 (27.5)	−1.41 (0.91)	51.44	50.65	43.24	40.17	3.48	3.46	2.35	2.33
8	0 (27.5)	1.41 (0.99)	49.98	47.68	65.34	64.32	4.58	4.46	3.22	3.13
9	0 (27.5)	0 (0.95)	87.50	87.37	87.40	87.42	4.54	4.53	3.43	3.55
10	0 (27.5)	0 (0.95)	87.67	87.50	87.59	87.60	4.61	4.57	3.44	3.62
11	0 (27.5)	0 (0.95)	87.40	87.73	87.76	87.75	4.51	4.51	3.46	3.26

Coded variables, −1.41, −1, 0, +1, +1.41; real variables are in parentheses; a_w: water activity; 10288: *Aspergillus carbonarius* CCDCA 10288; 10293: *Aspergillus carbonarius* CCDCA 10293, 10211: *Aspergillus ochraceus* CCDCA 10211; 10212: *Aspergillus ochraceus* CCDCA 10212.

(IPCC, 2007). The optimistic scenario suggests an increase of 1.15 °C in the average temperature whereas the pessimistic scenario suggests an increase of 3.7 °C. After the coffee is harvested, its moisture content is around 30–50% (Borém, Reinato, & Andrade, 2008) which corresponds to an a_w greater than 0.9 (Iaccheri et al., 2015; Siqueira et al., 2017). Water activity remains around 0.9 until humidity reaches 20% bu (Iaccheri et al., 2015). This was chosen in the model to estimate the ORI, i.e., a_w 0.95 for *A. carbonarius* and 0.96 for *A. ochraceus*; these a_w values show the highest ORI.

3. Results

The effects of temperature and a_w on the growth and ORI of *A. carbonarius* CCDCA 10288 and 10293 and *A. ochraceus* CCDCA 10211 and 10212 were evaluated using a CCRD. The design matrix and the obtained results are shown in Table 1.

3.1. Evaluation of Aspergillus carbonarius and Aspergillus ochraceus growth at different temperatures and a_w

Through the obtained results, regression coefficients were calculated, and mathematical models were constructed. Table 2 shows the models, coefficients of determination R^2, and the statistical parameters. The percentage of variance explained by the models was satisfactory for all studied isolates. All designs were adjusted by a quadratic model with $R^2 > 0.90$. The *F* calculated value is higher than table *F* value ($F_{0.95\ 5;\ 27} = 2.57$), indicating that the model is predictive. In addition, the p values indicated significant regressions ($p \leq 0.05$). These models were used to construct the contour curves (Fig. 2) for visualizing the most appropriate conditions of temperature and a_w, which resulted in better growth of the isolates.

According to the contour curves (Fig. 1A and B), it was observed that the isolates of *A. carbonarius* CCDCA 10288 and 10293 showed the highest growth (78 mm) under the same conditions, with a_w between 0.935 and 0.965, and temperature between 25 and 32 °C. *A. ochraceus* CCDCA 10211 and 10212 showed the highest growth (81 mm), with a_w between 0.940 and 0.990 and temperature between 21 and 30 °C.

3.2. Evaluation of ORI for Aspergillus carbonarius and Aspergillus ochraceus at different temperatures and a_w

Through the obtained results, regression coefficients were calculated, and the mathematical models were constructed. Table 3 shows the models, coefficients of determination R^2, and the statistical parameters. The models were statistically significant at 90% confidence ($p < 0.10$). However, analysis of the coefficients of determination (R^2) indicates that isolate *A. ochraceus* 10212 showed the lowest values $R^2 < 0.60$; thus, the model cannot be used, and the surface is not generated for this isolate.

All designs were adjusted by a quadratic model with $R^2 > 0.70$ for *A. carbonarius* and *A. ochraceus* 10211. The F calculated value is higher than table F value ($F_{5;\ 5;\ 0.10} = 3.45$), suggesting that the model is predictive. In addition, the p values indicate significant regressions ($p \leq 0.10$). From these models, contour curves were constructed (Fig. 2).

Based on the contour curves (Fig. 2A), it was observed that the isolates of *A. carbonarius* CCDCA 10288 showed the highest ORI in a_w between 0.95 and 0.99 and temperature between 24 and 32 °C. *A. carbonarius* CCDCA 10293 (Fig. 2B) shows the highest ORI under the same conditions of a_w and temperature between 22 and 32 °C. *A. ochraceus* CCDCA 10211 in Fig. 2C showed the highest ORI at a_w between 0.97 and 0.99 and temperature between 25 and 30 °C.

Table 2
Models predicted for the growth of the evaluated isolates.

Fungi	Predicted model	R^2	F	p
A. carbonarius CCDCA 10288 (mm)	$Cres = -1.90 + 47.23T - 0.51T^2 + 3.89a_w - 2.02a_w^2 - 18.98Ta_w$	0.84	29.85	$3.3900e^{-10}$
A. carbonarius CCDCA 10293 (mm)	$Cres = -1.94 + 49.55T - 0.51T^2 + 3.96a_w - 2.05a_w^2 - 24.41Ta_w$	0.86	33.57	$8.9515e^{-11}$
A. ochraceus CCDCA 10211 (mm)	$Cres = -1.36 + 72.71T - 0.62T^2 + 2.65a_w - 1.32a_w^2 - 42.00Ta_w$	0.96	125.61	0
A. ochraceus CCDCA 10212 (mm)	$Cres = -1.49 + 64.71T - 0.61T^2 + 2.93a_w - 1.48a_w^2 - 34.16Ta_w$	0.98	122.44	0

3.3. Use of the predicted model to estimate ORI of Aspergillus carbonarius and Aspergillus ochraceus in the southern region of Minas Gerais

The model obtained in this study was used to estimate the ORI in the largest coffee producing region in Brazil. The temperature used in the model was the average monthly temperature of the region according to the INMET and IPCC, and the a_w used was defined according to the characteristics of the coffee during drying as described in Materials and Methods 2.7. Fig. 3 shows the results obtained by using the predicted model to estimate the ORI in each month during the year 2017. According to the results obtained, it is estimated that in all months of the year, there is a risk of OTA production by the species studied (Fig. 3). *A. carbonarius* CCDCA 10293 (Fig. 3B) showed the lowest values of ORI in relation to other isolates whereas *A. ochraceus* CCDCA 10211 (Fig. 3 C) showed the highest ORI. In relation to the current scenario, the highest ORI is found in the months of January to April and from September to December, the lowest values are in May to August for all isolates. In the optimistic scenario, there is an increase in ORI mainly in these months whereas for *A. carbonarius* CCDCA 10293, the increase is observed only in these months. The pessimistic scenario presents the same results with the increase being even higher.

4. Discussion

The present study evaluated the fungal growth conditions and OTA production; through the obtained model, a future prediction of the risk of mycotoxin production due to climatic changes was made.

Conditions of a_w and temperature are important limiting factors for growth and ochratoxin production and may vary depending on the substrate (Kokkonen, Jestoi, & Rizzo, 2005) and toxigenic fungal strain (Esteban, Abarca, Bragulat, & Cabañes, 2006; Kouadio et al., 2007; Mitchell, Aldred, & Magan, 2003). In this work no difference was observed between strains for growth. The best growth obtained was within the range already observed in other studies for *A. carbonarius* and *A. ochraceus* in coffee-based culture media, temperature around 30 °C, and a_w around 0.99 (Kouadio et al., 2007). The difference the ochratoxin production between the strains of *A. carbonarius* and *A. ochraceus* was verified.

OTA production was evaluated and used to calculate the OTA risk index (ORI) as proposed by Gil-Serna et al. (2014). ORI represents how much the studied condition is favorable for the growth and mycotoxin production in the fungus. Thus, if different strains produce the same amount of OTA and grow differently, the highest ORI will be presented by the highest growing strain. Its development will be more probable, and therefore will present a greater risk of being present and producing OTA in the studied condition.

The optimum conditions for OTA production by *A. carbonarius* in different substrates include temperatures between 15 and 25 °C and a_w of 0.95–0.99 (Alborch, Bragulat, Abarca, & Cabañes, 2011; Kapetanakou et al., 2009; Mitchell, Parra, Aldred, & Magan. 2004; Romero, Pinto, Patriarca, & Vaamonde, 2010). Souza et al. (2016) evaluated OTA production in synthetic coffee medium and observed that the highest OTA production by *A. carbonarius* was at 15 °C and 0.99

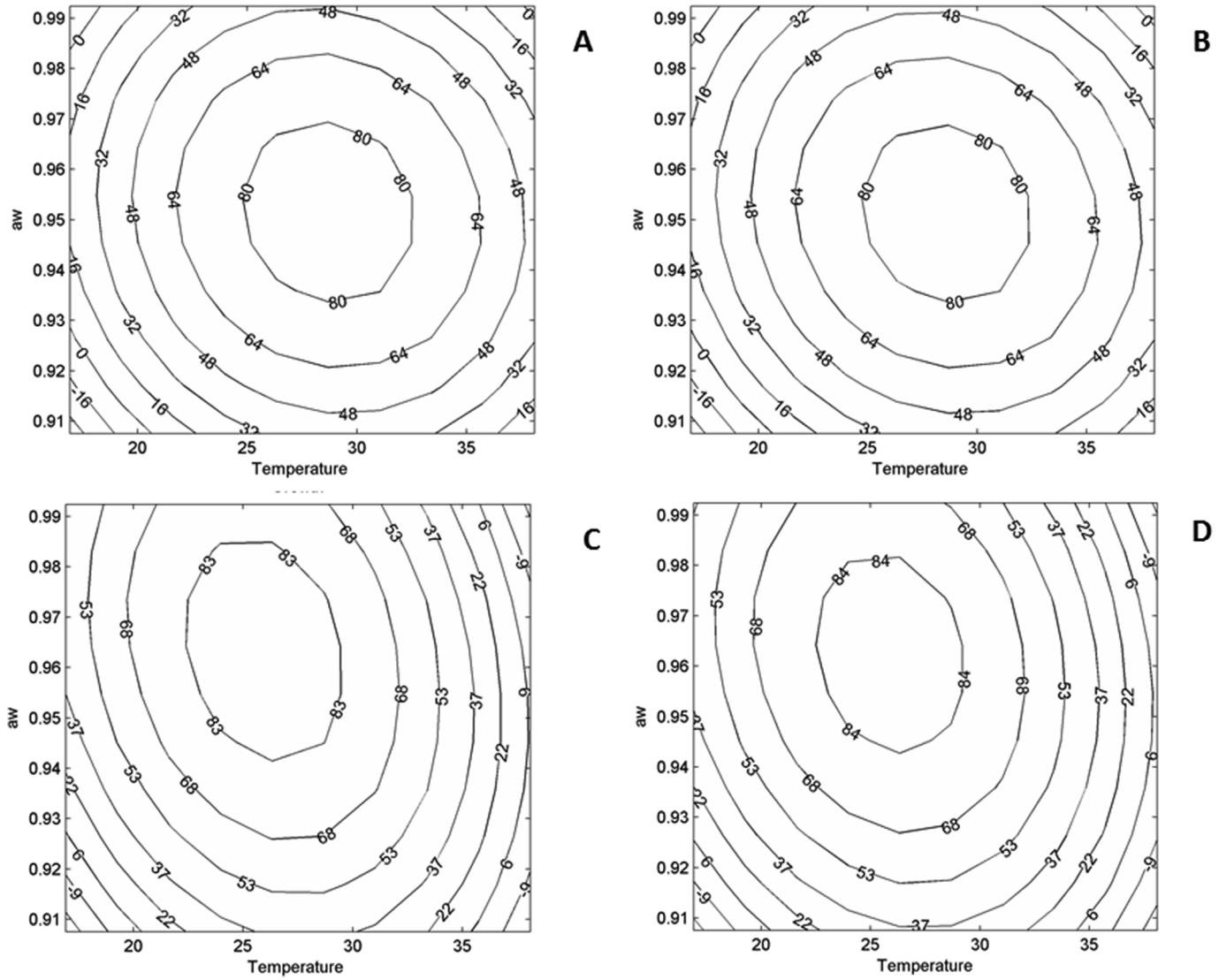

Fig. 1. Contour curve for growth as function of temperature and water activity for *Aspergillus carbonarius* CCDCA 10288 (A); *Aspergillus carbonarius* CCDCA 10293 (B); *Aspergillus ochraceus* CCDCA 10211 (C) and *Aspergillus ochraceus* CCDCA 10212 (D).

a_w, which is within the range found for other substrates. In this work, the range of the highest ORI found for *A. carbonarius* in relation to a_w is close to the described works, but the temperature range is higher. The method of risk assessment that takes growth into account presents the highest ORI near the range of its optimal growth. In relation to *A. ochraceus*, the highest risk found coincided with the highest OTA production reported in previous studies. Optimum conditions for OTA production by *A. ochraceus* generally include temperatures between 12 and 37 °C (optimal condition at 25 °C) and from 0.85 a_w to the optimal 0.97 a_w. (Moss, 1991). Souza et al. (2016), evaluated OTA production in synthetic coffee culture medium, and observed that two isolates of *A. ochraceus* had higher OTA production at 25 °C and 0.99 a_w.

The optimal condition of a_w and temperature for ORI, shown by *A. carbonarius* and *A. ochraceus* correspond to the conditions in the coffee bean when processing begins (when the coffee is placed in the yard for drying). At this stage of processing the producer must take utmost care to avoid fungal contamination. Correspondence between laboratory tested a_w and that measured on a sun-dried ripe cherry batch shows that the first 5 days of drying are critical for fungal growth and OTA production (Kouadio et al., 2007). The average infection rates for cherries taken from trees are very low. During processing, the point after harvesting presents a higher risk of contamination by filamentous fungi (Silva et al., 2008). A high frequency of *A. carbonarius* and *A. ochraceus* is present in the drying yard and ground and where the highest levels of OTA are also found (Batista et al., 2009; Pitt, Taniwaki, & Cole, 2013; Taniwaki et al., 2003).

It has been suggested that climate change factors will have a profound impact on sustainable food production systems (Akbar et al., 2016). Considering that fungi can be found in coffee and have potential for mycotoxin production, it is important to assess the effect of climate change on this potential.

According to the model obtained in this work and using the estimated temperatures it was possible to predict the risk of OTA production during the year according to the climate change scenario. The months that presented the lowest ORI coincide with the harvest season in Brazil, which is usually from May to August. However, with climate changes, and especially in the pessimistic scenario, there is an increase in the ORI in these months. Under these scenarios, there is a high risk of coffee becoming grossly contaminated with fungi and showing greater OTA production. Therefore, considering the climatic changes, the producer should take greater care since this risk would be higher especially at the harvest season. The temperature increase conditions will favor the growth and OTA production of the fungi studied mainly for *A. ochraceus*, and the ORI will be high.

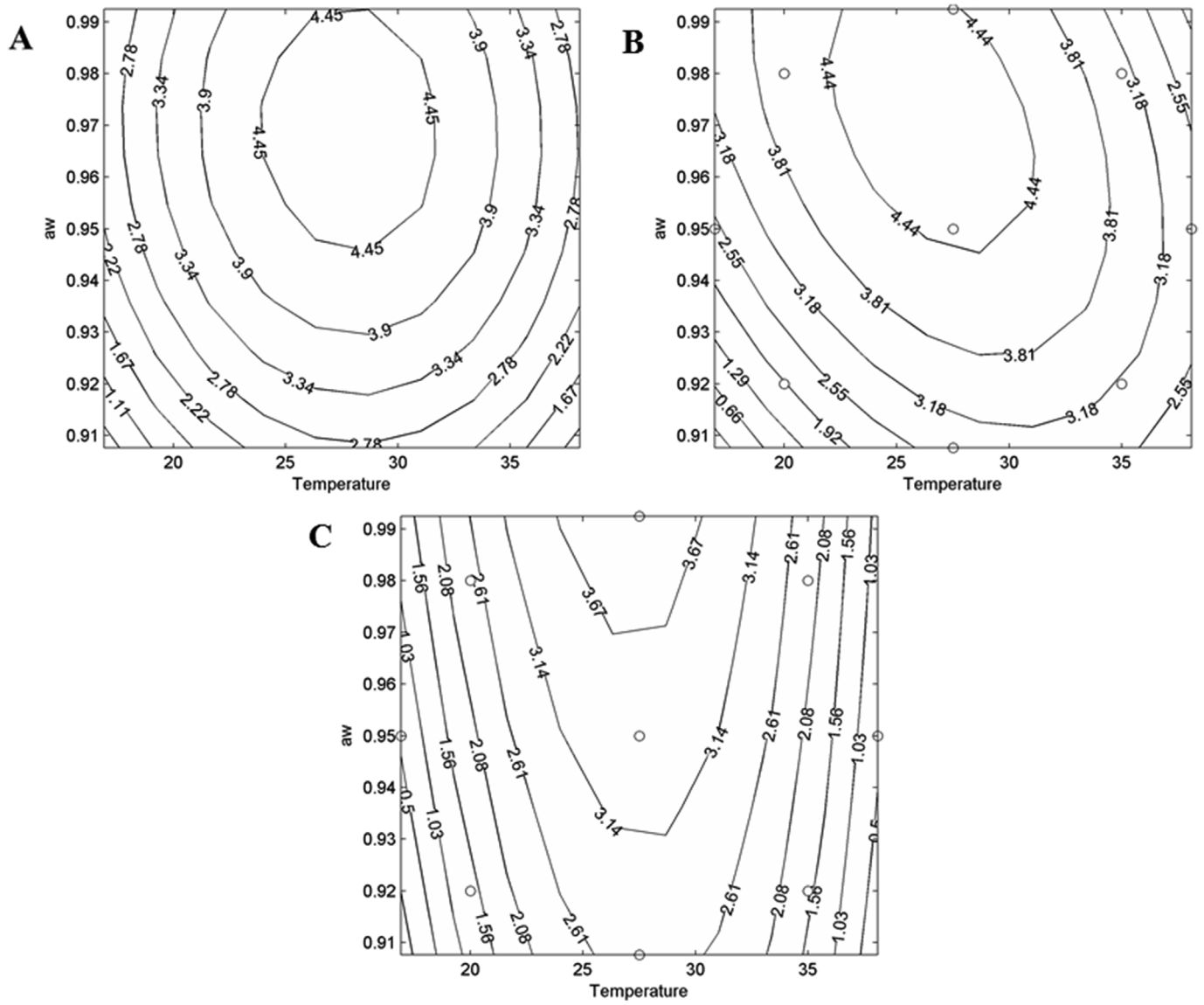

Fig. 2. Contour curve for ORI as function of the interactions between temperature and water activity for *Aspergillus carbonarius* CCDCA 10288 (A); *Aspergillus carbonarius* CCDCA 10293 (B) *Aspergillus ochraceus* CCDCA 10211 (C).

The producer must follow good practices when putting coffee on the yard in thin layers. Another important procedure would be to remove defective grains because they are more associated with the presence of fungi and mycotoxin production (Barcelo, Barcelo, & Alvarez, 2017; Taniwaki, Teixeira, Teixeira, Copetti, & Iamanaka, 2014). Another alternative that has been studied is the use of inoculants (such as yeast) that inhibit the presence of ochratoxigenic fungi (Gil-Serna, Patino, Cortes, Gonzalez-Jaen, & Vazquez, 2011; Ramos, Silva, Batista, & Schwan, 2010; Souza et al., 2017; Velmourougane, Bhat, Gopinandhan, & Panneerselvam, 2011). Following some of these practices, the producer can inhibit the presence of the fungus and consequently the OTA production.

5. Conclusions

The conditions under which the species showed the highest risk of

Table 3
Models predicted for the ORI of the evaluated isolates.

Fungi	Predicted model	R^2	F	P
A. carbonarius CCDCA 10288	$ORI = -518.30 + 1.31T - 0.02T^2 + 1042a_w - 533.80a_w^2 - 0.26Ta_w$	0.79	3.75	0.086
A. carbonarius CCDCA 10293	$ORI = -533.48 + 3.06T - 0.02T^2 + 1024a_w - 496.08a_w^2 - 2.22Ta_w$	0.87	6.88	0.027
A. ochraceus CCDCA 10211	$ORI = -78.42 + 1.84T - 0.02T^2 + 105.25a_w - 41.43a_w^2 - 0.47Ta_w$	0.83	4.92	0.050
A. ochraceus CCDCA 10212	$ORI = 56.27 + 2.26T - 0.02T^2 - 196.64a_w + 129.64a_w^2 - 1.09Ta_w$	0.55	0.02	0.902

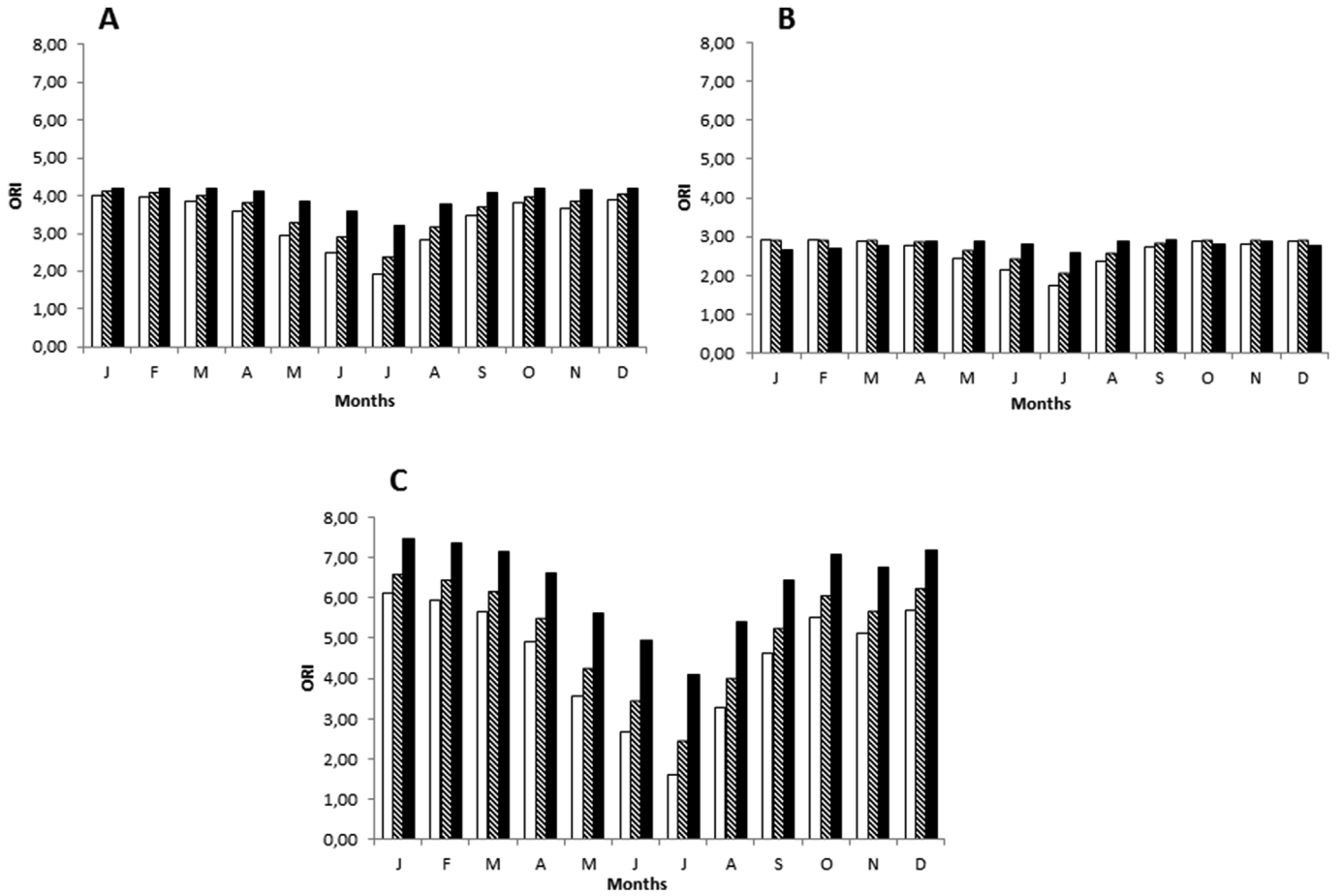

Fig. 3. ORI in each month during the year 2017 in relation to the current average temperatures (□) temperatures of optimistic scenario (▨) and temperatures of pessimistic scenarios (■) for *Aspergillus carbonarius* CCDCA 10288 (A); *Aspergillus carbonarius* CCDCA 10293 (B) *Aspergillus ochraceus* CCDCA 10211 (C).

OTA production were as follows: a_w between 0.95 and 0.99 and temperature between 24 and 32 °C for *A. carbonarius* CCDCA 10288; a_w between 0.95 and 0.99 and temperature between 22 and 32 °C for *A. carbonarius* CCDCA 10293; and a_w between 0.97 and 0.99 and temperature between 25 and 30 °C for *A. ochraceus* CCDCA 10211. Through the models obtained, it was possible to predict that with the climatic changes, the risk of mycotoxin production will increase mainly for the species *A. ochraceus* in the southern region of Minas Gerais. Thus, producers should maintain good agricultural practices during the processing of coffee in order to avoid contamination and OTA production by fungi.

Conflicts of interest

There are no conflicts of interest to declare.

Declarations of interest

None.

Acknowledgments

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